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First record of *Russula anthracina* and its ectomycorrhiza associated with Himalayan cedar from South Asia

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Abstract — *Russula anthracina* and its ectomycorrhiza are identified on the basis of morphological characters and molecular analysis. The species is characterized by brittle and blackening basidiomata with pinkish lamellae. Ectomycorrhizal morphotypes are characterized by monopodial pinnate ramification and the presence of typical russuloid cystidia on outer mantle surface. Molecular phylogenetic analysis clustered the ITS sequences generated in this study with other *R. anthracina* sequences. This is the first report of *R. anthracina* from Pakistan and South Asia.

Key words — *Cedrus deodara*, *Russula* sect. *Compactae*, Swat

Introduction

The genus *Russula* Pers. (*Agaricomycetes, Russulales*, *Russulaceae*) is represented by more than 750 species distributed worldwide (Kirk et al. 2008, Das et al. 2013, Arora & Nguyen 2014, Razaq et al. 2014, Dutta et al. 2015). From Pakistan, 26 species of *Russula* have been reported to date (Ahmad et al. 1997, Niazi et al. 2006, Niazi 2008, Razaq et al. 2014, Jabeen et al 2015). *Russula* species are important ecologically as ectomycorrhizal symbionts associated with many kinds of forest trees (Richardson 1970; Bills et al. 1986; Villeneuve et al. 1989, 1991; Gardes & Bruns 1996; Buyck et al. 1996). Many species are harvested worldwide for consumption (Fogel 1975, Fogel & Trappe 1978, Hu & Zeng 1992, Guo 1992, Rammeloo & Walleyn 1993, Buyck 1994).

Russula anthracina is characterized in *R. sect. Compactae*, which consists of blackening species. Taxa in the section are quite easy to identify as a group.

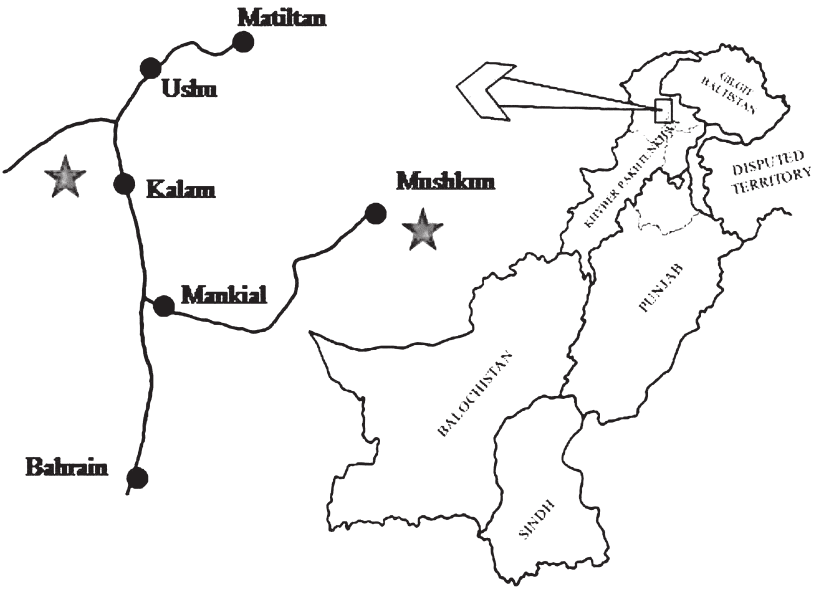


Fig. 1. Map of Pakistan showing sampling sites.

Their squat, robust, and brittle basidiomata are initially white but rapidly become brown to black in all parts (Kibby 2001). However within the section species are less easily distinguished due to their intergrading colors and obscure morphological and anatomical variations. Here, combining morphological and molecular analyses greatly helps in the correct identification of taxa (Arora & Nguyen 2014).

The goal of the current study was to collect ectomycorrhizal fungal fruit bodies and their ectomycorrhizae associated with Himalayan cedar from Pakistan. During the field survey (2012–14) in monsoon season, abundant *Russula anthracina* fruiting bodies were found in coniferous forests of Swat district, Khyber Pakhtunkhwa, Pakistan. The area is surrounded by mountains and has a typical dry temperate climate (Stucki & Khan 1999). The mountains are dominated by forests of *Cedrus deodara* (Roxb. ex D. Don) G. Don along with *Pinus* spp. and *Quercus oblongata* D. Don [= *Q. incana* Roxb., nom. illeg.] (Champion et al. 1965). *Russula anthracina* constituted the most abundant macrofungal taxon observed during 2013 in Kalam (Fig. 1), a lush green area along the river Swat. *Russula anthracina* fruiting bodies and ectomycorrhizae associated with Himalayan cedar were also collected from Mushkun (Fig. 1).

Nuclear ribosomal DNA sequence analyses of the ITS region from fruit bodies and ectomycorrhizal tissues were combined with detailed morphological examination to confirm our Pakistani collections as *R. anthracina*.

Materials & methods

Collection, morphological and anatomical study of basidiomata

Specimens were photographed in the field using a Nikon D70S digital camera. Fresh morphological characters were recorded and colors were designated using Munsell Color System (1975) after which the specimens were preserved by drying over hot air. For detailed anatomical examination, tissues from lamellae, pileipellis and stipitipellis were mounted on glass slides and observed in Phloxine (1%) for better contrast, Melzer's reagent for amyloid basidiospore ornamentation, and KOH (5%) for colored hyphae using a Meiji Techno MX4300H microscope. Anatomical features were measured using a Carl Zeiss Jena ocular micrometer and line drawing were made using a Leitz Wetzlar camera lucida.

The abbreviation 'n/b/p' indicates 'n' basidiospores measured from 'b' basidiomata from 'p' collections. Basidiospore dimensions are given as length × width (l × w), with extreme values given in parenthesis. Q = l/w ratio of individual spores; avQ = average Q of all spores ± standard deviation. Collector's numbers prefixed with K refer to Kalam and SJ (basidiomata) and SA (ectomycorrhizae) refer to sequence codes. Voucher specimens were deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH).

Collection, isolation, morphological and anatomical study of ectomycorrhiza

15-cm^3 soil blocks were dug a few centimetres away from tree trunks using extreme care to insure that the fine roots in the soil block belonged to the selected tree species. Twenty soil cores were taken from each sampling site. In laboratory, each soil core was soaked in water for few hours to loosen the adhering soil particles and then put on a 2 mm sieve under running water to separate the roots from the soil. The ectomycorrhizae (EcM) were carefully sorted out into morphotypes under incandescent light and then under a Meiji Techno EMZ-5TR stereomicroscope. The morphotypes were cleaned under the stereomicroscope using a fine brush. Morphologically identical morphotypes were kept in McCartney bottles in distilled water for later examination. Replicates of these morphotypes were kept at 8 °C in eppendorf tubes containing 1 mL of 2% CTAB buffer for molecular analysis. Morphological characters were noted under the stereomicroscope. For anatomical details, mantle layers (inner and outer) and radiating hyphae were mounted in trypan blue stain (Agerer 1991). Hyphae were measured using an ocular micrometer and drawn using a camera lucida.

DNA extraction, amplification and sequencing

For genomic DNA extraction, 2 mg of fungal tissue was taken from the gills in an eppendorf tube, 2–4 ectomycorrhizal root tips were taken in a separate eppendorf tube. DNA was extracted following modified CTAB method (Bruns 1995). Using forward primer ITS1F (5′-CTTGGTCATTTAGAGGAAGT-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990), internal transcribed spacer (ITS)

regions along with the central 5.8S region of nuclear ribosomal DNA (nrDNA) were amplified following Gardes & Bruns (1993). The PCR products were sent to Macrogen Inc. (Korea) for sequencing.

Molecular phylogenetic analysis

Consensus sequences were generated from the sequences obtained by both primers in BioEdit software. These sequences were then BLAST searched at NCBI (http://www.ncbi.nlm.nih.gov/). Sequences with closest match were selected from GenBank to reconstruct phylogeny (sequences with less query cover and negative E values were not included). Published sequences of the most closely related species were also included in the final data set. *Boletus reticuloceps* (M. Zang et al.) Q.B. Wang & Y.J. Yao was chosen as outgroup (Shimono et al. 2004). Multiple sequences were aligned using online MUSCLE by EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/muscle/). The sequences were trimmed with the conserved motifs 5′-(…GAT)CATTA… and …GACCT(CAAA…)-3′ and the alignment portions between them were used for phylogenetic analysis. Maximum likelihood (ML) analysis was performed using General Time Reversible model (Nei & Kumar 2000) in MEGA6 software (Tamura et al. 2013) to test the phylogeny at 1000 bootstraps. Percentage identity and divergence in nrDNA-ITS of the taxa were analysed using MegAlign (DNAStar, Inc.). Sequences generated in this study were submitted to GenBank.

Russula anthracina Romagn., Bull. Mens. Soc. Linn. Lyon 31: 173 (1962) Figs 2, 3

Basidiomata medium to large. Pileus 4.5–8.6 cm broad, depressed from the middle; black to greyish black (10YR1/2) to grey (10YR2/2), dark brown (10YR2/4) to light brown (10R3/6) and pinkish (5YR9/2) in the form of random patches; surface dry, smooth, reflective; margins smooth, incurved when young becoming straight at maturity. Lamellae ≤0.3 cm broad, pinkish (5YR9/2), regular, adnexed, subdistant to close, edge entire. Lamellulae frequent, single-tiered, variable in length. Stipe 3.3–5.1 × 1.1–2.5 cm, white, central, cylindrical, slightly narrower towards the apex. Surface smooth to finely wrinkled.

Basidiospores [n/b/p = 40/4/2] (5.4–)7.3–8.0(–8.3) × (4.7–)6.1–7.0(–7.8) µm, Q = 1.03–1.23, avQ = 1.12 ± 0.04; globose to subglobose, echinulate, echines ≤0.2 µm; apiculus prominent; guttulate, pale yellow in 5% KOH, amyloid in Melzer's reagent. Basidia 40.6–66 × 9.7 – 10.8 µm, clavate, wall ≤3 µm, pale yellow in 5% KOH, guttulate. Pleurocystidia 68.4–110 × 5.2–5.9 µm, fusoid, guttulate, pale yellow in 5 % KOH. Cheilocystidia 52.7–71 × 5.4–6.4 µm, fusiform, guttulate, pale yellow in 5% KOH. Pileipellis hyphae 3.8–5.1 µm wide, filamentous, branched, pale yellow to transparent in 5% KOH, septa infrequent, clamp connections and pileocystidia not observed. Stipitipellis hyphae 3.1–4.7 µm diam., filamentous, branched, hyaline to pale yellow, rarely septate, clamp connections not observed.

Fig. 2. *Russula anthracina* basidiomata. A. LAH35009; B. LAH35008. Scale bars = 0.5 cm.

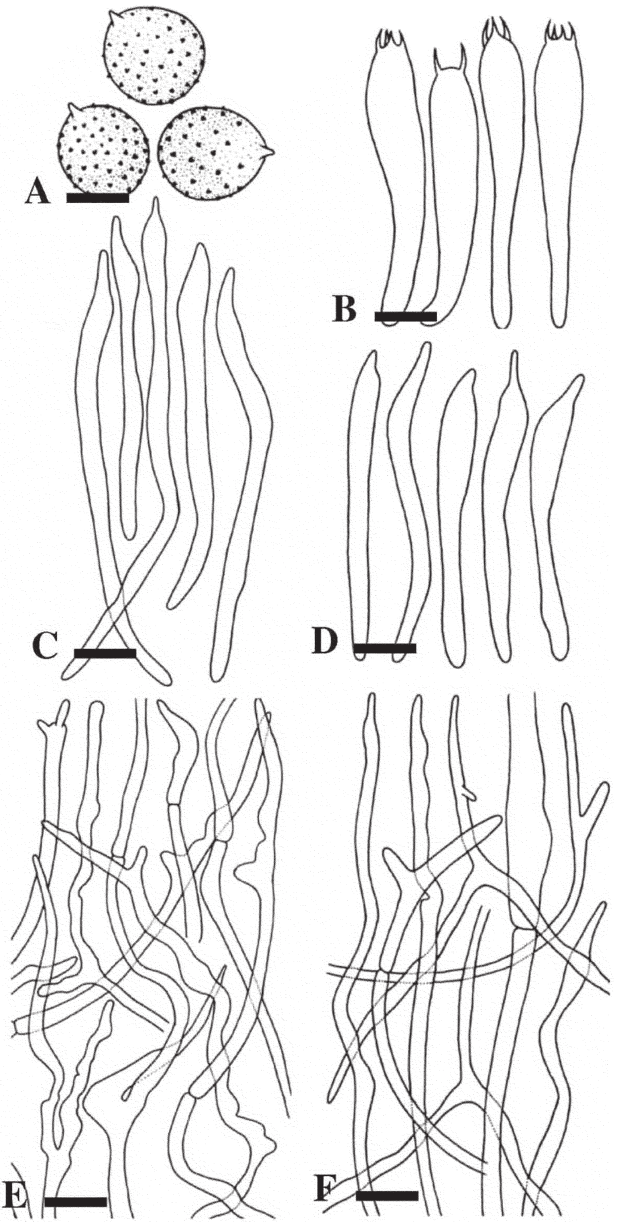


Fig. 3. *Russula anthracina* (LAH35008). A. basidiospores; B. basidia; C. pleurocystidia; D. cheilocystidia; E. hyphae from stipitipellis; F. hyphae from pileipellis. Scale bars: A = 4.5 µm; B, F = 12 µm; C = 20.5 µm; D = 10 µm; E = 14 µm.

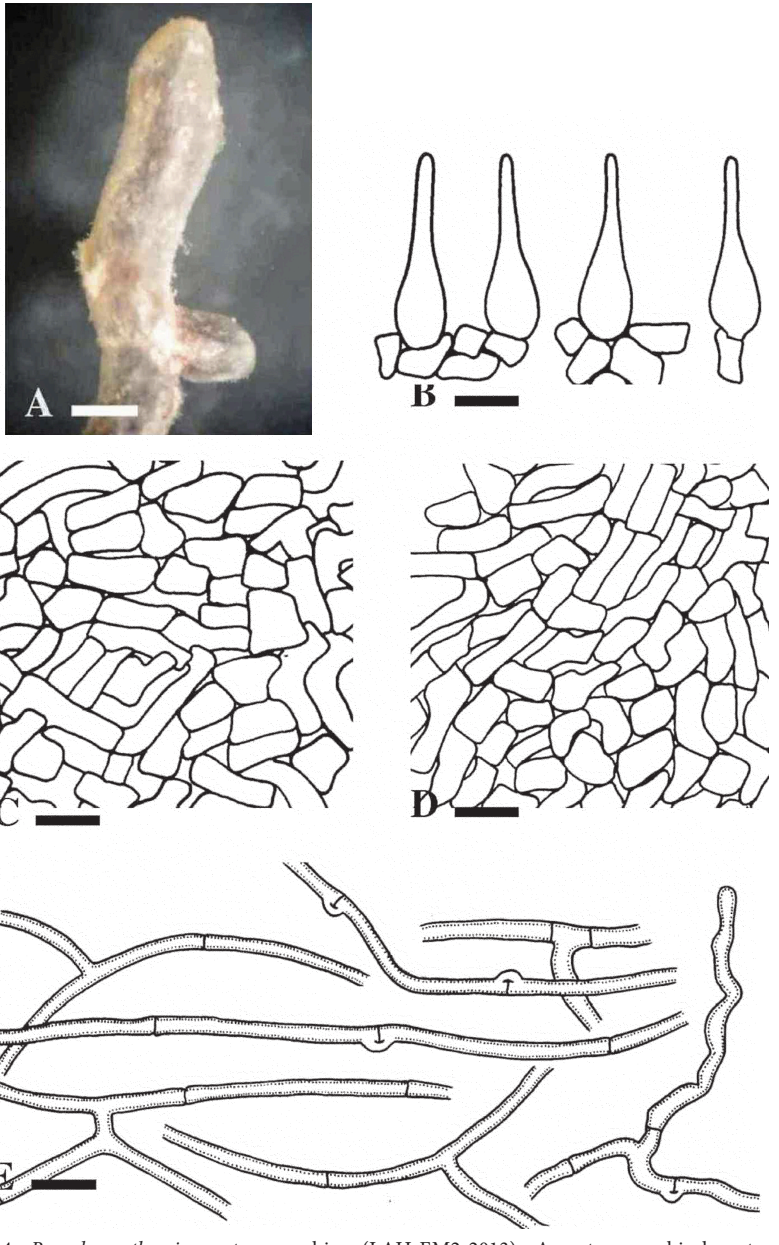


Fig. 4. *Russula anthracina* ectomycorrhiza (LAH-EM2-2013). A. ectomycorrhizal system; B. cystidia; C. inner mantle layer; D. outer mantle layer; E. emanating hyphae. Scale bars: A = 0.7 mm; B = 8.5 µm; C = 12 µm; D = 14.5 µm; E = 25 µm.

Material examined: **PAKISTAN**, **Khyber Pakhtunkhwa Province**, Swat District, Kalam, 2070 m asl, on soil under *Cedrus deodara*, 3 Sep 2013, Sana Jabeen K3; SJ5 (LAH35008; GenBank KR011879); Mushkun, 2500 m asl, under *Cedrus deodara*, 5 Sep 2013, Sana Jabeen K4-22; SJ19 (LAH35009; GenBank KR011880).

Morphological characterization of EcM **Fig. 4**

Ectomycorrhizal system monopodial pinnate, 0.7–1.2 cm long, axis ≤1 mm diam., unramified ends straight, cylindrical, greyish brown (10YR7/4) to dark brown at maturity, tip greyish brown (10YR7/4). Mantle greyish brown to transparent, distinct, smooth surface, lustre matte, cortical cells visible under the mantle surface at some tips. Rhizomorphs not observed. Emanating hyphae transparent, rare, sometimes frequent at few root tips, straight, cylindrical rarely branched. Cystidia frequent.

Outer mantle layer cells 14.6 × 8.1 µm, inner mantle layer cells 12.9 × 6.1 µm. Cells in both layers pseudoparenchymatous, yellowish to transparent, angular to somewhat roundish or elongated (mantle type L; Agerer 1987–2002), contents clear.

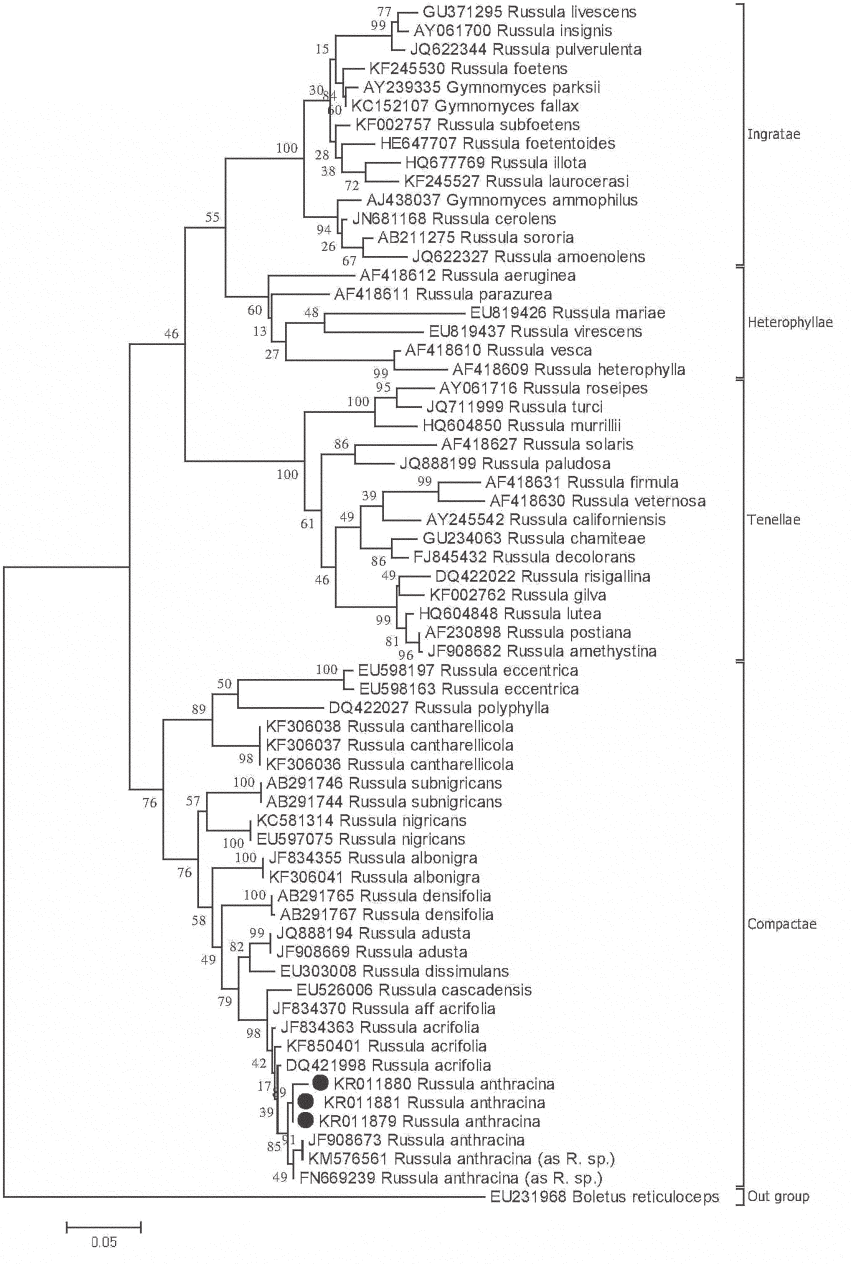
Emanating hyphae elongated, transparent, straight, cylindrical, ≤3.9 µm in diameter, wall ≤0.7 µm thick, surface smooth, septate, septa frequent, 2.0–49.3 µm apart, clamp connections rare, ornamentation absent, contents clear, ramification rather frequent, Y-shaped, anastomoses H-shaped, frequent, without septa and clamp connections. Cystidia lageniform, frequent, 23.9 µm long, strongly inflated base, ≤6.8 µm diam., attachment point ≤5 µm wide, neck straight, 4.9 µm diam. with 3.7 µm tip.

Material examined: **PAKISTAN**, **Khyber Pakhtunkhwa Province**, Swat District, Mushkun, 2500 m asl, associated with *Cedrus deodara*, 5 Sep 2013, Sana Jabeen K4-5; SA99 (LAH-EM2-2013; GenBank KR011881).

Molecular phylogenetic characterization **Fig. 5**

Sequencing of the *Russula anthracina* PCR products using ITS1F/ITS4 yielded 697–752 base pairs. A 598-base pair consensus sequence was obtained by trimming the motifs. BLAST of *R. anthracina* (KR011881) revealed it as 99% identical with KF679819, FN669239, and KM576561 (100% query cover, 0.0 E value) and a 99% identity with JF908673 (93% query cover,

Fig. 5. Molecular phylogenetic analysis of *Russula* spp. inferred by the Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search was obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated by the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 64 nucleotide sequences. New sequences generated from Pakistan are marked with ●. There were a total of 828 positions in the final dataset.


GU371295 Russula livescens
AY061700 Russula insignis
JQ622344 Russula pulverulenta
KF245530 Russula foetens
AY239335 Gymnomyces parksii
KC152107 Gymnomyces fallax
KF002757 Russula subfoetens
HE647707 Russula foetentoides
HQ677769 Russula illota
KF245527 Russula laurocerasi
AJ438037 Gymnomyces ammophilus
JN681168 Russula cerolens
AB211275 Russula sororia
JQ622327 Russula amoenolens
AF418612 Russula aeruginea
AF418611 Russula parazurea
EU819426 Russula mariae
EU819437 Russula virescens
AF418610 Russula vesca
AF418609 Russula heterophylla
AY061716 Russula roseipes
JQ711999 Russula turci
HQ604850 Russula murrillii
AF418627 Russula solaris
JQ888199 Russula paludosa
AF418631 Russula firmula
AF418630 Russula veternosa
AY245542 Russula californiensis
GU234063 Russula chamiteae
FJ845432 Russula decolorans
DQ422022 Russula risigallina
KF002762 Russula gilva
HQ604848 Russula lutea
AF230898 Russula postiana
JF908682 Russula amethystina
EU598197 Russula eccentrica
EU598163 Russula eccentrica
DQ422027 Russula polyphylla
KF306038 Russula cantharellicola
KF306037 Russula cantharellicola
KF306036 Russula cantharellicola
AB291746 Russula subnigricans
AB291744 Russula subnigricans
KC581314 Russula nigricans
EU597075 Russula nigricans
JF834355 Russula albonigra
KF306041 Russula albonigra
AB291765 Russula densifolia
AB291767 Russula densifolia
JQ888194 Russula adusta
JF908669 Russula adusta
EU303008 Russula dissimulans
EU526006 Russula cascadensis
JF834370 Russula aff acrifolia
JF834363 Russula acrifolia
KF850401 Russula acrifolia
DQ421998 Russula acrifolia
KR011880 Russula anthracina
KR011881 Russula anthracina
KR011879 Russula anthracina
JF908673 Russula anthracina
KM576561 Russula anthracina (as R. sp.)
FN669239 Russula anthracina (as R. sp.)
EU231968 Boletus reticuloceps
Ingratae
Heterophyllae
Tenellae
Compactae
Out group
0.05

0.0 E value). Published sequences from different *Russula* sections were included to reconstruct a phylogeny with *Boletus reticuloceps* (EU231968) as outgroup. The analysis revealed four major clades (/compactae, /heterophyllae, /ingratae, /tenellae) corresponding with the taxonomic sections (Fig. 5). Species within these clades were clustered with strong bootstrap, supported by morphological characters. Within /compactae, *R. acrifolia* Romagn., *R. adusta* (Pers.) Fr., *R. albonigra* (Krombh.) Fr., *R. anthracina*, *R. cantharellicola* Arora & N.H. Nguyen, *R. cascadensis* Shaffer, *R. densifolia* Secr. ex Gillet, *R. dissimulans* Shaffer, *R. eccentrica* Peck, *R. nigricans* Fr., *R. polyphylla* Peck, and *R. subnigricans* Hongo clustered with 76% bootstrap support.

Discussion

Russula anthracina can be distinguished from *R. acrifolia* by its pinkish tinged lamellae (Romagnesi 1967, Galli 1996, Sarnari 1998), which are pale cream in *R. acrifolia*. Anatomically, in *R. anthracina* the pileus cuticle lacks pileocystidia, which are numerous with frequently diverticulate tips in *R. acrifolia*. *Russula albonigra* is distinguished from *R. anthracina* and *R. acrifolia* by its rapidly blackening flesh and rather large, unforked pilocystidia (Rayner 1985, Kibby 2001).

Russula anthracina is an ectomycorrhizal symbiont of both deciduous and coniferous trees (Gminder et al. 2000, Bahram et al. 2011, Suz et al. 2014). We describe and illustrate this ectomycorrhiza here for the first time in detail. Morphologically it differs from the ectomycorrhiza of the closely related *R. acrifolia* (Agerer 1996) by its monopodial pinnate branching pattern and greyish brown to transparent mantle surface with cellular hyphae and typical russuloid cystidia without an apical knob.

In phylogenetic analysis, our Pakistani sequences showed a 0.2–1% genetic divergence from *R. acrifolia* and clustered with European *R. anthracina* sequences from Austria, Estonia, and Italy with 85% bootstrap support. *Russula anthracina* is a European species (Romagnesi 1967, Petersen & Vesterholt 1993, Einhellinger 1994, Hallingbäck & Aronsson 1998, Gminder et al. 2000, Piltaver et al. 2002, Legon & Henrici 2005, Kränzlin 2005, Adamčík et al. 2006, Holec & Beran 2006, Dimou et al. 2008, Ronikier & Adamčík 2009, Bahram et al. 2011, Cabo 2012, Kasom & Karadelev 2012, Osmundson et al. 2013, Suz et al., 2014), but it has also been recorded in America (Roberts 2007, Kuo 2009) and Africa (El Kholfy et al. 2011, Kinge et al. 2013). From western Asia, it is reported from Turkey (Yalz et al., 2006, Günç Ergönül et al. 2013, Sesli & Denchev 2014). Our *Russula anthracina* collections represent an addition to the mycobiota of Pakistan and a first report of the species from South Asia.

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